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A novel conformation of the LC3-interacting region motif revealed by the structure of a complex between LC3B and RavZ

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ABSTRACT

LC3-family member proteins play a critical role in autophagy, a cellular process responsible for the degradation of massive cellular components including intracellular pathogens. A variety of molecules involved in the autophagic pathway engage in specific interactions with a unique sequence motif referred to as the LIR (LC3-interacting region) motif. Although identification of conserved structural features of LIR motifs in complex with LC3-family members has established a canonical LIR motif, atypical conformations of LIR motifs have recently been revealed. Here, we determined the three-dimensional crystal structures of LC3B in complex with three different LIR motifs of RavZ from *Legionella pneumophila*, an intracellular pathogen that can manipulate the host autophagy system. The tandem LIR motifs located in the N-terminal region of RavZ adopt a novel β-sheet conformation and thus provide specific ionic interactions with LC3B in addition to canonical hydrophobic plugged-in interactions. Consequently, these motifs possess higher binding affinity to LC3-family members than canonical LIR motifs, although the tandem repeats can only bind to one LC3 molecule. These findings broaden our understanding of the functional repertoire of LIR motifs in autophagy.

1. Introduction

Macroautophagy (hereafter referred to as autophagy) is a conserved degradation pathway utilized for the removal of unnecessary or damaged intracellular components and organelles in eukaryotic cells [1,2]. Autophagy was initially thought to be a non-specific process, however a wide variety of recent studies investigating selective autophagy have emerged [3–6]. In contrast to non-selective autophagy, selective autophagy regulates the abundance of specific cargo molecules *via* autophagy receptors that target protein complexes, aggregates, invading microbes, and whole organelles into lysosomes [6–8]. These specific autophagy receptors link the autophagosome with target molecules, and thus interact with both target cargo and Atg8-family member proteins embedded on the autophagosomal membrane. It is known that ubiquitylation plays a critical role in marking specific target molecules, and ubiquitylated cargo are recognized by the ubiquitin binding domain of autophagy receptors [5]. All of these receptors have an LC3-interacting region (LIR) motif to facilitate targeting to the autophagosomal membrane.

Of the various cargo targeted by autophagy, intracellular bacterial pathogens represent one type of substrate that autophagy targets for degradation [9–12]. When infected by bacteria, host cells utilize the autophagy system to clear the invading pathogens. The process related to autophagic clearance of intracellular pathogens is referred to as “xenophagy”. To survive xenophagy, various pathogens have evolved strategies to block or overcome host-mediated autophagy. In particular, *Legionella pneumophila* utilizes the effector protein RavZ to inhibit xenophagy [13]. The RavZ protein secreted by *Legionella pneumophila* can localize onto the LC3-conjugated phagophore membrane and cleaves the C-terminus of phosphatidylethanolamine (PE)-conjugated Atg8-family member proteins such as LC3 and GABARAP. Similar to other ubiquitin-like (Ubl) proteins, Atg8-family member proteins possess a β-grasp domain and a short flexible C-terminal tail that typically ends with at least one glycine residue [14–17]. Furthermore, they are

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distinguished by the presence of one or two α-helices near their N-terminus, which are crucial for providing the interaction site for the LIR motif [18,19]. RavZ cleaves the peptide bond between a hydrophobic residue and the terminal glycine of PE-conjugated LC3. As a result, *Legionella pneumophila* can effectively inhibit xenophagy by removing LC3 from the autophagosomal or phagophore membrane [13,20–24].

LC3 recognizes the LIR motif containing the Ω-x-x-Ψ sequence, where Ω and Ψ represent aromatic and hydrophobic residues, respectively, and the two inner residues (-x-x-) can be any amino acid residue [25,26]. In general, the presence of one or more acidic residues are necessary immediately before the LIR motif. In our previous report [22], distinct LIR motifs at the N- and C-terminal tails of RavZ were identified. Using these LIR motifs, RavZ is able to bind two LC3 molecules separately. Intriguingly, the N-terminal tail of RavZ contains mysterious tandem LIR repeats (LIR1 and LIR2), while the C-terminal tail of RavZ possesses a canonical LIR sequence (LIR3). More recently, there is a report that the structure of RavZ-LC3 shows non-functional complex as authors described and that of LIR2-LC3 fusion protein displays a canonical binding mode [23,24]. However, the characteristics of the N-terminal tandem repeats of the LIR motif remain unclear. This paper provides the first description of structural details and a novel conformation adopted by the tandem repeat LIR motif. In contrast to the canonical LIR motif, the N-terminal tandem LIR repeats of RavZ are maintained by a specific β-sheet conformation. Furthermore, we determined that the unique conformation of the LIR motif can bind to LC3B more tightly than the canonical motif, suggesting that RavZ overwhelms the interaction between LC3B and intracellular proteins containing LIR motifs. These results shed light on our understanding of the manner by which bacterial effector proteins overcome host autophagy mechanisms at the molecular level.

2. Materials and methods

The details of materials and methods are in the supplementary material.

3. Results

3.1. Each N-term and C-term LIR peptide binds to LC3B with 1:1 stoichiometry

RavZ possesses three LIR motifs at flexible regions (Fig. 1A), and the N- and C-terminal tails of RavZ interact with LC3B separately as shown in our previous report [22]. However, it is unclear why tandem LIR motifs at the N-terminal region interact with only one LC3B. To clarify the role of this tandem repeat of LIR motifs, we performed SEC-MALS using RavZ LIR peptides (NLIR: residues 12–34 and CLIR: residues 429–438) and LC3B (Fig. 1B). Although two successive LIR motifs are present in the NLIR peptide of RavZ, the binding stoichiometry of NLIR and LC3B was determined to be 1:1, the same as for CLIR and LC3B. It indicated that two LC3B molecules are unable to bind simultaneously to the NLIR peptide. In an effort to characterize the feature of this NLIR of RavZ, structural studies of LC3B in complex with LIR peptides were initiated.

3.2. Structure of LC3B in complex with N-terminal tandem LIR motifs of RavZ

As the LIR motifs of RavZ possess intrinsic flexibility and low

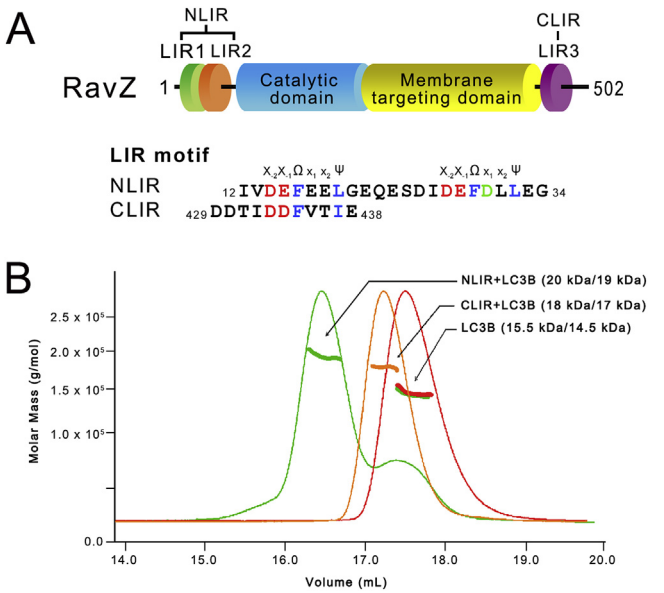

Fig. 1. Interaction between RavZ LIR peptides and LC3B. (A) Domain architecture of RavZ indicating the location of three LIR motifs (LIR1, LIR2 and LIR3). The abbreviations of each peptide used in this study are shown. Sequence of LIR motifs are aligned and the consensus motif X_{-2}-X_{-1}-Ω-x_1-x_2-Ψ (Ω: aromatic ring; Ψ: hydrophobic; x_n and X_n (n = 1, 2): any amino acid) is indicated. The critical hydrophobic phenylalanine and leucine (or isoleucine) residues in the F-x_1-x_2-L(I) motif are colored blue and the preceding 2 acidic residues (X_{-2} and X_{-1}) are colored red. (B) The LC3B with NLIR (green line), LC3B with CLIR (orange line), and LC3B alone (red line) were analyzed by SEC-MALS. The horizontal line represents the measured molecular mass (MM). Excess LC3B with MM of 15 kDa elutes later. Each species is indicated by an arrow with experimental (MALS) and theoretically calculated (Calc) MM values shown in parentheses (MALS/Calc). This shows the 1:1 binding stoichiometry between RavZ peptides (NLIR and CLIR) and LC3B. Sequence information of each peptide is given in panel (A). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

binding affinity to LC3B, we failed to determine the structure of the RavZ-LC3B or LIR peptide-LC3B complexes. Instead, we generated the chimeric protein NLIR-LC3B ($RavZ^{12-34}$ + $MAP1LC3B^{2-120}$), which has been widely used for crystallization of the LC3B-LIR complex [27–29]. The structure of NLIR-LC3B was determined at 1.88 Å resolution. The overall structure of LC3B in the complex is virtually the same as previously reported LC3 structures, however, the structure of bound LIR differs from previously reported LIR structures (Fig. 2A). Each β-strand formed by residues belonging to the first LIR (LIR1) and second LIR (LIR2) motifs makes an anti-parallel β-sheet connected by a loop mimicking the shape of a horseshoe (Fig. 2A). LIR1 is bound to the pocket of LC3B whereas the second LIR motif (LIR2) does not bind to the pocket of LC3B. The LIR1 motif possesses the sequence X_{-2}-X_{-1}-Ω-x_1-x_2-Ψ (Fig. 1A), where Phe16 is located at the Ω (aromatic ring) position and Leu19 at the Ψ (hydrophobic) position. In general, the two residues (x_1 and x_2) between the characteristic aromatic and hydrophobic residues can be any amino acid residue since these do not participate in LC3B binding, although Glu18 at position x_2 in RavZ participates in LC3B binding (Fig. 2B and C). Two acidic residues Asp14 and Glu15 at positions X_{-2} and X_{-1}, respectively, provide additional ionic interactions with positively charge residues of LC3B, which is quite common in the LIR-LC3B interaction [25,30]. As noted, the unusual secondary structure of the LIR2 motif is not directly involved with the hydrophobic binding pocket of LC3B, however, it provides an

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